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Document downloaded from:

<http://hdl.handle.net/10459.1/65396>

The final publication is available at:

<https://doi.org/10.1016/j.envexpbot.2017.09.010>

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## **Circadian regulation of photosynthesis and transpiration from genes to ecosystems**

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## **Highlights**

- Circadian rhythms control 15-35% of diurnal photosynthesis and stomatal conductance
- Effects across phylogenies studied so far are consistent, but important gaps remain
- New methods allow quantification of circadian regulation in trees and ecosystems
- Circadian gas exchange regulation is adaptive to environmental variation

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2    **ecosystems**

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## 13    **Abstract**

14    Circadian regulation is an endogenous self-sustaining mechanism that drives temporal  
15    gene expression and, amongst others, affects the diurnal patterns of photosynthesis  
16    ( $A$ ) and stomatal conductance ( $g_s$ ). Here we review current knowledge on how  
17    circadian regulation drives diurnal gas exchange from genes to ecosystems in the  
18    field. Molecular mechanisms underlying the structure of circadian clocks and how  
19    they regulate  $A$  and  $g_s$  in a few model species are starting to be elucidated but  
20    additional data are required to understand regulation across phylogenies, especially  
21    within the gymnosperms, and across environments and scales. Circadian rhythms  
22    were responsible for 15-25% and for 30-35% of the daytime oscillations in  $A$  and  $g_s$ ,  
23    respectively, across the C3 and C4 species for which data are available. Consequently,  
24    circadian effects over diurnal gas exchange are of similar magnitude to the effects of  
25    temperature or vapor pressure deficit. Moreover, recent findings indicate how  
26    circadian rhythms could exert significant impacts on ecosystem patterns of gas  
27    exchange, which would challenge conventional approaches to derive the  
28    environmental flux dependences. Progress in transferring laboratory findings to the  
29    field is being hampered by lack of suitable experimental and modeling facilities that  
30    can disentangle circadian effects from environmental responses in the field and in  
31    ecosystems, and methodological recommendations are offered. The effects of  
32    environmental stressors on circadian regulation of gas exchange are also poorly  
33    understood. We document how circadian control of gas exchange may be adaptive by  
34    allowing plants to anticipate highly predictable environmental cues, but also by  
35    increasing the diversity of potential gas exchange responses to environmental  
36    variation in plant populations.

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38    **Keywords:** biosphere-atmosphere interactions, circadian clock, ecology, ecosystem,  
39    non-model species, stomatal conductance.  
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## 41    **1. Introduction**

42    The Earth rotates on its axis every day and around the sun every year. Day and night  
43    transitions and photoperiodic oscillations vary deterministically as a function of time  
44    and location and, consequently, constitute the most predictable environmental cue.  
45    These cyclic oscillations, repeated for a few billion years, have influenced life  
46    through the evolution of circadian clocks, amongst others (Pittendrigh, 1981). The  
47    circadian clock is an endogenous subcellular mechanism that allows organisms to tell  
48    the time and to consequently adjust their metabolism in advance of predictable  
49    environmental cues, such as dawn and dusk transitions.

50            The discovery of circadian rhythms predates that of photosynthesis and is  
51    often attributed to de Mairan (1729), who observed continuous nyctinastic movements  
52    under protracted darkness. Circadian regulation in photosynthesis in C3 “higher”  
53    plants was first described by Hillman (1971) (although it had been previously  
54    described in algae), and first measurements of circadian regulation in stomatal  
55    aperture were provided by Mansfield and Heath (1963) in the dark and by Martin and  
56    Meidner (1971) in the light. A large body of literature documenting circadian  
57    regulation in gas exchange has developed over the last five decades, but most of this  
58    work has concentrated in a few model species and within lab settings, where  
59    environmental conditions can be controlled straightforwardly.

60            Understanding diurnal variations in photosynthesis and transpiration in the  
61    field has also been the subject of considerable research in the last few decades, but  
62    this work has mostly focused on understanding direct physiological responses to  
63    temperature, radiation and other changes in the physical environment over the day and  
64    the night. The effect of circadian regulation over diurnal patterns of field gas  
65    exchange has traditionally been considered negligible. However, recent studies on the

ecological relevance of circadian rhythms indicate how circadian regulation could explain up to 30% of the diurnal variation in net CO<sub>2</sub> exchange (A) and 70% in stomatal conductance ( $g_s$ ) at leaf and at whole canopy scales during a 24-h cycle (Resco de Dios et al., 2016a), and how the effects of circadian regulation over the temporal pattern of nighttime  $g_s$  could be equal or more important than the effects of vapour pressure deficit (Resco de Dios et al., 2013a; Resco de Dios et al., 2013b).

In this manuscript, we will review our current knowledge on circadian regulation of photosynthesis and transpiration in C3 and C4 plants, taking trees and other woody species into particular account. More specifically, we seek to synthesize our current knowledge on how circadian regulation affects diurnal gas exchange in the field at leaf, canopy and ecosystem scales. We will first explain what the circadian clock is and what are its mechanistic underpinnings. Next, we will describe the mechanisms by which circadian regulation affects transpiration and then the mechanisms underlying circadian regulation of photosynthesis. Within these sections we provide an account of the species where circadian transpiration and photosynthesis have been examined and discuss how the mechanisms may vary across phylogenies. We will furthermore discuss different methods to measure circadian regulation in the field and we will explain why current methods used by molecular biology cannot be readily applied. We will then focus on how to model circadian regulation and on why it is necessary to study the role of circadian clocks as drivers of gas exchange under field conditions. Finally, we will show the adaptive potential for circadian regulation of gas exchange. The review is based upon the articles that have been published on “circadian AND stomata\*” and “circadian AND photosynthesis”, according to Web of Science (search strings entered on 5<sup>th</sup> July 2017). Ultimately, we hope that our review will help in bridging the gap between molecular studies on clock action,



mainly performed under lab conditions, and ecological studies on diurnal gas exchange including old-grown trees and forest ecosystems.

## **2. What is the plant circadian clock**

The circadian clock regulates the temporal pattern of expression in ~30% of the genome in the model plant *Arabidopsis thaliana* in a manner that is independent, to some extent, of environmental fluctuations (Michael et al., 2008). The end-result is a rhythmic oscillation in various aspects of metabolism. For instance, upon exposure to constant environmental conditions of light, temperature, etc. for a few days, an oscillation in gas exchange, amongst other processes, with a 24-h period becomes apparent (Fig. 1a). Moreover, circadian oscillations are temperature-compensated, meaning that the period is preserved across different temperatures and can be phase-shifted by light. Circadian rhythms are sometimes mistaken for diurnal variations (King et al., 2013), but the word *circadian* implies the presence of a self-sustaining oscillator with a period of approximately (*circa*) 24-h (*dies*). Detailed reviews on the structure of circadian clocks have been recently published (Greenham and McClung, 2015; Hernando et al., 2017; Millar, 2016). Here we seek to provide an introductory view on circadian clocks that provides a basic understanding for field plant or ecosystem scientists.

The circadian clock was initially viewed as having three different components: an input system, providing environmental information; a central oscillator, composed of the “canonical clock genes” and that constitutes the core structure of the clock; and the outputs, comprising the clock-driven downstream processes (Lakin-Thomas, 2001) (Fig. 1b). The central oscillator is composed by different transcription–translation feedback loops, whereby the canonical clock genes are rhythmically

transcribed and translated into proteins that feedback to inhibit their own transcription. In its current model, the transcription-translation feedback loops conforming the central oscillator consist of a ring of four repressors (quadrirepressilator) with some transcriptional activators (Fig. 1c) (Hernando et al., 2017; Millar, 2016).

The expression of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY) increases from midnight until reaching a peak at dawn, and inhibit the transcriptional induction of PSEUDORESPONSE REGULATOR 5 (PRR5) and TIMING OF CAB EXPRESSION 1 (TOC1). The expression of PRR7 and PRR9 increase during the morning and inhibit expression of LHY and CCA1, ending the morning phase. In turn, falling levels of CCA1 and LHY allow for expression of EARLY FLOWERING 3 (ELF3), ELF4 and LUX ARRHYTHMO (LUX), the so-called evening complex, with expression peaks before dusk. The evening complex then inhibits expression of the PRRs and ends the day phase. Additionally, the transcriptional regulators associated to the photoreceptors NIGHT LIGHT INDUCIBLE AND LOCK REGULATED 1 and 2 (LNK1 and LNK2) promote the expression of PRR5, TOC1 and ELF4 and, in turn, PRRs and TOC1 bind to LNK promoters and inhibit their expression (Hernando et al., 2017; Millar, 2016). Such a complex molecular structure is thought to be a requirement for allowing accurate response under varied, real-life photoperiodic oscillations (Troein et al., 2009). Additionally, inputs (light and temperature signaling) and outputs (metabolism) interact with the central oscillator such that different metabolic reactions (such as photosynthesis) are both, masters and slaves to the clock (Shin et al., 2017).

Circadian clocks occur in every cell individually. In fact, coordination across circadian clocks within an organ is more driven by external cues than by internal communication signals (Wenden et al., 2012). Consequently, circadian clocks within an organism or organ may show contrasting phases and, for instance, guard cell clocks show different phases than mesophyll clocks (Endo, 2016; Yakir et al., 2011). While circadian clocks for stomatal conductance have been documented to be independent from circadian rhythms in photosynthesis (Dodd et al., 2004; Hennessey and Field, 1991), there is some hierarchical structure in plant clocks (Takahashi et al. 2015). For instance, the downward flow of photosynthates serves to entrain the root clock, which acts as a slave to the shoot clock (James et al., 2008). Similarly, the clock in the vascular tissue communicates with and regulates the clock in the mesophyll (Endo, 2016).

Circadian regulation controls the plant metabolism by, at least four different processes (Greenham and McClung, 2015; Yakir et al., 2007): i) some of the canonical clock genes are transcription factors (e.g.: CCA1, LHY) and, consequently, they regulate the temporal pattern of transcription related to the output processes; ii) the oscillator exerts controls some post-transcriptional processes such as controlled protein turnover, alternative splicing and chromatin modification, which further adjust metabolism; iii) circadian regulation controls hormone expression, to some degree, and circadian outputs may be indirectly controlled via hormone activity; iv)  $\text{Ca}^{2+}$  is a messenger for different processes and circadian oscillations in  $\text{Ca}^{2+}$  may trigger temporal patterns of metabolism.

### **3. Circadian regulation of transpiration**

#### ***3.1. Mechanisms***

Mencuccini et al. (2000) observed how, for a given level of leaf water potential and of abscisic acid (ABA) concentrations,  $g_s$  was lower in the afternoon than in the morning in common bean. This process, where the response to a stimulus depends on the time of day, is termed “circadian gating” and may be explained by a reciprocal interaction between TOC1 and ABA (Legnaioli et al., 2009). The expression of TOC1 is induced by ABA and gated by the circadian clock, such that maximum sensitivity to ABA occurs in the subjective afternoon and leads to stomatal closure. TOC1 in turn affects ABA concentrations by inhibiting the expression of ABAR (ABA receptor protein) (Fig. 2), but the underlying mechanism is less well understood. Circadian gating may not be the only process underlying diurnal changes in the sensitivity to ABA, as diurnal changes in pH or in cytokinin concentration could also affect the response (Correia et al., 1997).

Contrary to conventional wisdom, an increasing body of literature is indicating how stomata do not stay constantly closed overnight (Caird et al., 2007; Forster, 2014). On the contrary,  $g_s$  often shows a temporal pattern of increasing values from midnight to dawn that cannot be explained by variation in vapor pressure deficit and other environmental drivers alone (Caird et al., 2007; Resco de Dios et al., 2013b). In fact, predawn stomatal opening may also be explained by circadian gating as increasing expression of CCA1/LHY overnight, which represses TOC1 activity, could lead to increases in predawn stomatal priming (Pokhilko et al., 2013).

Morning stomatal opening is also mediated by interactions between circadian regulation and FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF) (Ando et al., 2013; Kinoshita et al., 2011). Null mutants of *ft* and *tsf* show smaller rates of  $g_s$  than the wild-type and it has been proposed that FT and TSF effects on  $g_s$  are mediated by the clock gene ELF3 (Fig. 2a). As previously noted, we solely seek to

provide here an initial description of the molecular processes explaining circadian regulation of  $g_s$ . More detailed information may be found elsewhere (Hubbard and Webb, 2015).

In addition to direct effects over stomatal conductance, circadian regulation could also affect transpiration by affecting leaf and root hydraulic conductances (Caldeira et al., 2014) and thus water transport within the plant. In fact, aquaporin expression, being an important regulator of plant hydraulics (Kaldenhoff et al., 2008), is also one of the clock outputs, which could explain why the temporal pattern in leaf hydraulic conductance in sunflower took a few days to recover when the pattern of light-dark cycles reverted under experimental conditions (Nardini et al., 2005).

### 3.2. *Species*

Analyses on the evolution of circadian clocks indicate their prevalence among plant clades. However, presence of canonical clock genes does not necessarily imply that circadian expression will occur, or that circadian regulation across all possible outputs will occur. For instance, circadian clocks appear silent in reindeers, which may be advantageous as they live in environments where day/night oscillations are weak during much of the year (Lu et al., 2010). In our compilation of the plant literature, we have found compile direct evidence for circadian regulation in stomatal conductance across 27 species that belong, predominantly, to the categories of crops and to woody life forms such as arctic shrubs and tropical tree species, and with a few additional studies on temperate trees (Table 1; woody plants are displayed in bold). We were able to find reports of non-circadian regulation in  $g_s$  for only two tropical species. It is possible that there are more studies noting a lack of circadian regulation in  $g_s$  that remained unpublished due to publication bias (Dwan et al., 2008).

Using the studies displayed in Table 1, we digitized  $g_s$  patterns in the free-run period (continuous light and temperature) when available and documented the amplitude of circadian oscillations in  $g_s$  during the “subjective daytime” (the part of the diurnal cycle when it would have normally been daytime before subjecting the plants to continuous illumination). The amplitude of the response, normalized based on the maximum value measured under that radiation level, ranged from 30% in *A. thaliana* to 53% in *M. indica*. The generality of these data need to be interpreted with caution as they are based on a small number of species (Fig. 3). However, the values of the amplitudes are large enough to indicate a potentially significant influence of circadian regulation over daily  $g_s$  and that additional studies should be conducted to more fully quantify circadian effects across more species and functional groups. Still, the studies conducted so far provide information for species with highly contrasting phylogenetic origins within the angiosperms, indicating how effects might be widespread at least within this group. We did not take into consideration studies on species for which stomatal aperture (instead of  $g_s$ ) was assessed for elaboration of Table 1, but we do not expect that stomatal aperture will have been assessed in a large number of additional species. Importantly, Brinker et al. (2001) examined and documented a circadian rhythm in stomatal aperture in *Ginkgo biloba* in what remains, to date, the only study on a gymnosperm that we are aware of.

Lack of data on circadian regulation in  $g_s$  in gymnosperms is particularly problematic because the mechanisms regulating  $g_s$  are different from those in angiosperms. In particular, the relationship between ABA and  $g_s$  varies between both groups and, within the gymnosperms, important differences are also present between conifers and other clades such as cycads (McAdam and Brodribb, 2015). As previously documented, circadian regulation in  $g_s$  is, at least partly, driven by ABA. If

ABA is a more important driver of diurnal stomata in angiosperms than in gymnosperms (McAdam and Brodribb, 2015) that could imply that circadian regulation of  $g_s$  will be smaller in the latter. However, although circadian regulation of  $g_s$  in *Arabidopsis* (the angiosperm species, where most information is available) is partly dependent on ABA, it is possible that the mechanism by which circadian regulation acts on  $g_s$  in conifers is different.

## **4. Circadian regulation of photosynthesis**

### *4.1. Mechanisms*

Photosynthesis rates depend on the conductance of CO<sub>2</sub> diffusion through the stomatal pore (stomatal conductance) and from the substomatal cavity through the mesophyll to the chloroplast (mesophyll conductance), on the one hand, and on biochemical processes in the Calvin cycle on the other. Considering that  $g_s$  and mesophyll conductance ( $g_m$ ) are both regulated by the clock, it would seem feasible to hypothesize that circadian regulation in  $A$  results from circadian regulation of diffusional resistances.  $g_s$  regulates  $A$  by altering the concentration of CO<sub>2</sub> in the intercellular spaces ( $C_i$ ). However, studies conducted in free-run report a negative correlation between  $A$  and  $C_i$  (Doughty et al., 2006), or a lack of correlation between both variables (García-Plazaola et al., 2017) for some species, implying that circadian regulation in  $g_s$  is unlikely to drive circadian photosynthesis. A potential role for circadian  $g_m$  rhythms in regulating CO<sub>2</sub> concentrations in the chloroplast ( $C_c$ ) remains to be tested, to the best of our knowledge. Since aquaporins are involved in short-term changes in  $g_m$  (Flexas et al., 2012) and there is indication that aquaporin expression is clock controlled, there is good reason to assume circadian variation in  $g_m$  (Brilli et al., 2013). Additional structural features such as cell wall thickness, which do not show

diurnal variation, also influence  $g_m$  (Peguero-Pina et al., 2012). It is, however, likely that  $g_m$  similarly to  $g_s$  exerts a limited circadian influence on  $A$ .

Circadian oscillations in the composition of the light harvesting complexes are a more likely candidate for circadian oscillations in  $A$ . Rates of chlorophyll synthesis are under circadian control and diurnal fluctuations in Chl  $a/b$  have been linked with circadian oscillations in  $A$  and  $g_s$  in bean and cotton (García-Plazaola et al., 2017). These authors interpreted the oscillations in Chl  $a/b$  as indicators of the synthesis and/or degradation of the light-harvesting complex II, which varies in anti-phase with the Chl  $a/b$  ratio. Peaks in Chl  $a/b$  occurred during the subjective night, indicating minimal light harvesting capacity at that time and maximal expression of the light harvesting complex II during noon. This would indicate that circadian regulation seeks to maximize  $A$  during noon, and not to avoid photodamage, at least for non-stressed plants as they were used in the experiment reported. The underlying molecular mechanism could be related to the activation of the LIGHT-HARVESTING CHLOROPHYLL A/B PROTEIN (*LHCB*) gene which is mediated by CCA1 and LHY by binding directly to their promoters (Meehan et al., 1996; Wang and Tobin, 1998). Other processes, such as non-photochemical quenching (a proxy for the rate of energy dissipation), also show circadian oscillation, but they are considered to be a consequence, and not a cause, of photosynthetic oscillations (García-Plazaola et al., 2017).

Variations in carbon fixation may also be involved in circadian regulation of  $A$  but, instead of resulting from a single specific pathway, it would appear that multiple processes are involved (Dodd et al., 2014). For instance, circadian regulation regulates the transcription of genes associated with the Calvin cycle (Farré and Weise, 2012) and transitory starch reserves (Graf et al., 2010) and leaf starch concentrations



have been shown to vary in a circadian manner under constant light (Gessler et al. 2017). Moreover, photosynthates have also been shown to interact with the canonical clock gene PRR7 to regulate entrainment and maintenance of robust circadian rhythms (Haydon et al., 2013). However, most studies on circadian regulation of gas exchange have been measured under light limitation and, therefore, the potential role of circadian regulation in the “dark reactions” as drivers of oscillations in the actual rates of  $A$  at leaf levels is likely to have been underestimated.

#### 4.2. Species

Circadian regulation in photosynthesis has been examined in 40 species overall, which is a larger number of species compared to the assessment of circadian stomatal rhythms (Table 1). As previously noted, our literature search revealed that there are 3 times more studies on circadian regulation of photosynthesis (534 publications) than of stomatal conductance (173 publications). Interestingly, circadian rhythms seem to play a larger role in influencing  $g_s$  than  $A$ . The relative diurnal oscillation driven by the clock in  $A$  ranged between 15-25% across species (again normalized from maximum value), which is smaller than the relative change previously documented for  $g_s$  (30-53%). The differences in the amplitude of the oscillation between  $A$  and  $g_s$  lead to diurnal variations in intrinsic water use efficiency ( $W_i$ ), which follow species-specific patterns. Some species, like *M. indica*, show peaks in  $W_i$  in the subjective morning, but  $W_i$  in *P. vulgaris* and *L. esculentum* peak in the subjective late afternoon. Interestingly, minimum water use efficiency occurs at midday, which is consistent with the notion that circadian regulation seeks to maximize C assimilation.

From the 40 species studied, circadian regulation has been described in actual photosynthetic rates for 28 species, in chlorophyll fluorescence in 6 more species and

there were 6 tropical species for which no rhythm in  $A$  was apparent when exposed to constant environmental conditions. Similar to  $g_s$  studies, circadian  $A$  regulation has been documented in herbaceous species and grasses, arctic shrubs, tropical trees and a few temperate tree species. There is again a notable lack of studies in gymnosperms, where only circadian rhythms in chlorophyll fluorescence in *Ginkgo biloba* have so far been documented (Pavlovic et al., 2009).

It is well known for *Arabidopsis* that photosynthates are involved in driving the expression of the circadian clock in roots (James et al., 2008). Consistent with this idea, different studies on root respiration point towards circadian regulation in photosynthate allocation belowground as an important driver of the diurnal pattern of root respiration (Gavrichkova and Kuzyakov, 2016; Hirano et al., 2008). Moreover, recent studies associate circadian regulation of photosynthates transported to the root with diurnal changes in the composition of the root bacterial microbiome (Staley et al., 2017). For tall trees, it was observed that continuous sugar loading into and unloading from the phloem on the transport path causes a mixing of recent assimilates with stored carbohydrates (Gessler et al., 2014) and due to the long transport times along the trunk, the effects of circadian control of assimilate loading on belowground processes might be blurred. However, only recently it was observed for Douglas-fir, that root/soil respiration reacted rapidly to changes in canopy light availability pointing to a mechanism that works much faster than mass flow in the phloem would allow (Kayler et al., 2017). Such fast travelling pressure-concentration waves in the phloem, that are depending on the phloem loading in the leaves could still trigger diurnal changes in belowground processes over long distances. Over shorter distances, circadian rhythms in assimilation or photosynthate concentrations have also been linked with circadian rhythms in leaf respiration (Gessler et al., 2017).

## 5. Measuring circadian regulation in the field

Circadian regulation has often been assessed under controlled environmental conditions, in a single model species (*Arabidopsis*) and at molecular or leaf levels. However, the questions asked by molecular biologists or chronobiologists are often different to those relevant to field and ecosystem scientists. Moreover, the mechanisms as observed in *Arabidopsis* or even with tree seedlings under controlled conditions, might or might not be relevant for complex ecosystems such as forests. Consequently, current methods developed for assessing circadian regulation will not always be suitable for field experiments and alternatives may need to be sought. In this section, we outline the methods traditionally used, and then discuss how to adopt them for leaf-to-ecosystem level studies in the field.

The hallmark of circadian regulation is a self-sustained oscillation in the absence of environmental changes (free-run) that is maintained under different temperatures and that may be phase-shifted by light. Traditional measurements in chronobiology consequently involved measurements under different light-dark cycles (LD) where the period (T-cycle) varies (i.e.: short or long days, different hours for L and D in T-cycles) and is combined with experiments in the free-run (LL or DD)(Aschoff, 1981). Such experiments in canopies or ecosystems may be assessed in advanced experimental facilities (i.e. Ecotron, whole tree chambers (Fig. 4)), where environmental conditions are precisely controlled and real-time measurements of gas exchange are conducted at macrocosm or whole tree scales. These facilities allow for altering T-cycles and may provide the only option to conduct “pure” experiments on circadian regulation at canopy or ecosystem scales under field-like conditions. However, these facilities are rare - only a handful exist around the world - and tall

trees (the whole tree chambers at the at Western Sydney University are 9 m high) or even old-grown forests will not fit in. Consequently, only a limited number of studies may be conducted and, while they represent an excellent infrastructure to examine circadian effects, broad-scale testing of circadian regulation in the field requires additional methods.

Molecular studies on circadian regulation monitor the activity of different genes (either directly or using bioluminescence), proteins or metabolites, and/or use imaging to monitor leaf movements, chlorophyll fluorescence and/or reflectance during the different T-cycle experiments (Gould et al., 2009; Millar et al., 1992; Pan et al., 2015). Quantifications of phase, period and amplitude require that the monitoring occurs over several days to obtain enough replication (although auto-correlation from continuously measuring the same individual may lead to pseudo-replication). Moreover, to avoid “legacies” from environmental influences, quantification of rhythmic properties often begins in the second day in the free-run (Costa et al., 2013).

In contrast, leaf level circadian regulation in the field has been examined by enclosing an experimental leaf within the cuvette of an infra-red gas analyzer and measuring its gas exchange under continuous light while keeping the rest of the branch under darkness (Doughty et al., 2006; Mendes and Marengo, 2014). Problems associated with this technique include potential systemic signals from the rest of the plant (i.e.: changes in hydraulics as a result of changes in temperature or other environmental conditions, changes in source-sink relationships, etc) and the continuous enclosing of a leaf within a cuvette for 24-h also limits the potential for replication. Moreover, light in the field increases gradually in the morning and decreases gradually in the afternoon, whereas in growth chambers lights are usually

either on or off. Studies on circadian regulation in the laboratory often begin since time under continuous light in the early morning (“Zeitberger” time), whereas field studies typically begin at noon and monitor leaf activity under constant and high radiation. However, it would be nearly impossible to conduct such field studies by enclosing the leaf for more than 24-h within the cuvette and results with this method will therefore reflect a mix of circadian signals, systemic responses and environmental effects on endogenous processes.

With the popularization of molecular techniques, the use of genetically modified organisms, that show either knock-out mutations or overexpress the genes of interest has become widespread. For instance, mutants overexpressing CCA1 (*cca1-ox*) often lack rhythmicity, and represent a powerful “control” for testing circadian effects (Wang and Tobin, 1998). In field or ecosystem studies, however, the use of transgenic organisms would be challenging by the intrinsic complications of doing a targeted genetic modification in non-model organisms and the potential problems associated with releasing transgenic organisms into the wild. Furthermore, experimental mutants raise additional challenges such as potential pleiotropic effects (where modifying one gene may act on multiple processes, and not only the one of interest) and it could lead to changes in the behaviour of the organism to compensate for the mutation (Buckley, 2016). Consequently, gene manipulation experiments are often not recommended for field or ecosystem-level studies.

A powerful alternative to mutants are recombinant inbred lines (RILs), where self-fertilization for multiple generations leads to homozygous RILs (beneficial for studies on genotype  $\times$  environment interactions) and because RILs provide continuous genetic variation in the trait of interest (i.e. circadian period) from the two parental genotypes (Edwards et al., 2011). RILs have been used in assessments of the

genetic correlations between circadian regulation and gas exchange but, since multiple generations are necessary to establish RILs, they will be mostly limited to species with short life spans (Edwards et al., 2011) and are consequently mostly not an option for trees.

Measurements of circadian regulation in the field are thus logistically challenging. As a possible option to circumvent some of these problems, Resco de Dios et al. (2013a) proposed using the amplitude of the change in nocturnal conductance from midnight until predawn as an indicator of the amplitude of the circadian response. Assessing circadian regulation overnight is advantageous because the effect of the environment is often limited: there is no light and variations in temperature and in vapor pressure deficit are relatively smaller than during daytime. During cloudy nights, temperature and vapour pressure deficit variation are even mostly negligible. Such conditions represent a suitable environment to assess circadian effects, at least on nighttime processes (Resco de Dios et al., 2013a). Nocturnal stomatal conductance has been documented to show an increase from midnight until predawn and the increase is driven by circadian regulation (Caird et al., 2007; Resco de Dios et al., 2013a; Resco de Dios et al., 2015). Consequently, quantification of the amplitude of the change in  $g_s$  from midnight until dawn in cloudy nights presents a promising way forward. Additional studies will be necessary to quantify the relationship (if existing) between the amplitude of the nocturnal change in  $g_s$  and that in daytime  $g_s$  and  $A$ .

Assessments of the amplitude of circadian-driven increases in nocturnal  $g_s$  may be used for leaf-level studies and also in whole-plant or ecosystem approaches such as sap flux or lysimeters (Resco de Dios et al., 2015). The eddy covariance technique is widespread for examining diurnal patterns of gas exchange in ecosystems

(Fig. 4), but its use overnight will often be limited by lack of atmospheric turbulence (Baldocchi et al., 1988; Beringer et al., 2016). However, eddy covariance will provide continuous data of daytime net ecosystem exchanges of CO<sub>2</sub> and H<sub>2</sub>O although plant and soil fluxes are often confounded. Statistical filtering techniques that select data only under a given set of environmental conditions have been used to infer endogenous circadian regulation with some success (Doughty et al., 2006; Resco de Dios et al., 2012) and showed that this approach is also applicable to forests. These statistical filtering techniques could be accompanied by examinations of the temporal patterns of model residuals. In particular, the application of neural networks such as the Self-Organizing Linear Output model (SOLO) is a powerful approach (Hsu et al., 2002). SOLO provides an empirical fit to the data that is considered as the “best-possible” fit (Abramowitz et al., 2008) and examination of the temporal pattern of residuals delivers information on potential circadian effects. However, examination of the temporal pattern in more than one model should be performed whenever possible, as that would avoid problems associated with the particular form of the model. Additionally, co-variation between drivers and fluxes in eddy covariance could be analyzed separately for different timeframes (hourly/bi-hourly, Fig. 5), in an approach analogous to current efforts towards understanding temperature effects on fluxes under different light intensities (Clements et al., 2012).

Overall, there is a wealth of techniques possible to infer circadian regulation in the field including trees and forests. However, the impossibility of conducting leaf or ecosystem measurements for more than one day under continuous light implies that endogenous signals will represent a mix of purely circadian effects with endogenous “legacies from the recent past.” Consequently, using a diversity of techniques and combining field studies with growth chamber experiments represents a powerful way

forward. However difficult, examining circadian regulation in the field is a must. It is established that the expression of 10% of the mammal genome is clock regulated (Lowrey and Takahashi, 2011) and this has important implications for “real world” field responses. In plants, the circadian clock regulates ~30% of the entire genome (Michael et al., 2008), but its field implications remain largely unexplored. Moreover, circadian effects over physiology are context dependent, meaning that results from lab experiments will not always be replicated in the field (Edwards et al., 2016).

## **6. Modeling circadian regulation of gas exchange**

After measuring circadian regulation in the field, the next challenge lies in how to incorporate circadian regulation into gas exchange models. As mentioned above, circadian regulation has been mostly examined within lab settings, and that limits the degree of generality that may be drawn from environmental effects over the circadian clock. Moreover, circadian regulation has so far been largely described over molecular scales, but ecosystem gas exchange models require information at different scales. Given the lack of information on the mechanisms driving circadian gas exchange in the field, modeling efforts to date have been empirical.

Circadian regulation is considered to lead to time changing maximal potential values of  $A$  and  $g_s$  – i.e. the maximum rates can be different in the morning compared to the afternoon or evening. Consequently, the interaction between endogenous circadian regulation and environmental responses determines actual rates. Current models of circadian gas exchange assume that circadian effects have an additive interaction with the mean value of the parameter of interest such that (Resco de Dios et al., 2016a; Williams and Gorton, 1998):



$$Y = Y_m + Y_a \sin\left(Y_f + \frac{2\pi t}{24} + Y_p\right) \quad (\text{eq. 1})$$

Where  $Y$  will be parameter of interest and subscripts  $m$ ,  $a$ ,  $f$  and  $p$  indicate mean  $Y$  value, the amplitude, frequency and phase of the rhythm, respectively, and  $t$  is time in hours (since experimental onset). We exemplify usage by implementing circadian oscillators in the Medlyn et al. (2011) model of stomatal conductance:

$$g_s = g_0 + 1.6 \left(1 + \frac{g_1}{\sqrt{D}}\right) \left(\frac{A}{C_a}\right) \quad (\text{eq. 2})$$

where  $g_0$  and  $g_1$  are fitting parameters representing minimal conductance, and marginal water use efficiency and  $D$  and  $C_a$  are vapour pressure deficit and ambient  $\text{CO}_2$  concentrations, respectively. One could hypothesize that circadian oscillations are affecting  $g_0$ ,  $g_1$ , or both. Under the assumption that circadian oscillations affect  $g_1$ , implementing eq. 1 in eq. 2 leads to:

$$g_s = g_0 + 1.6 \left(1 + \frac{g_{1m} + g_{1a} \sin\left(g_{1f} + \frac{2\pi t}{24} + g_{1p}\right)}{\sqrt{D}}\right) \left(\frac{A}{C_a}\right) \quad (\text{eq. 3})$$

Similar approaches have been followed by others interested in circadian effects over hydraulic conductances (Tardieu et al., 2015). The assumption that circadian oscillators have an additive effect on gas exchange was recently validated in an Ecotron study (Resco de Dios et al., 2017). In this study, the diurnal variation in net  $\text{CO}_2$  canopy exchange (with an increase in temperature and vapour pressure deficit from 15°C and 0.6 kPa at 0600h to 30°C and 2.0kPa at 1400h, respectively leading to a 22% decrease in  $\text{CO}_2$  exchange) was equivalent to the sum of direct environmental responses (37% decrease during a ramped temperature and vapour pressure deficit

response curve over a short time period) and effects of circadian regulation (13% increase between 0600 and 1400h at constant temperature and vapour pressure deficit).

Different studies have obtained different results as to whether inclusion of circadian responses leads to improved model outputs. For instance, Williams and Gorton (1998) examined circadian regulation in *A* and observed a statistically significant increase in model fit after including circadian effects which they, however, deemed as too small and not being biologically significant. However, considering circadian regulation in stomatal conductance improved diurnal prediction by 8-17% in bean and cotton canopies (Resco de Dios et al., 2016a) but no data for tree species are available. Circadian regulation exerts a relatively larger role on  $g_s$  than on *A*, which could explain the differences across studies. However, additional factors could explain the limited increase in model fit in Williams and Gorton (1998), as they used an understory species, which will naturally tend towards lower amplitudes in circadian rhythmicity (Fig. 3) because understory species are exposed to smaller fluctuations in environmental conditions (Resco de Dios et al. 2016a).

A promising modeling approach was developed by Chew et al. (2014) who built a multi-scale model of growth and reproduction in *Arabidopsis*, and that considered circadian effects as part of the photoperiodic responses that induce flowering. The model did not include circadian regulation in gas exchange, but it represents a powerful platform to link molecular processes with whole plant and, potentially, ecosystem level responses that might also be applied to trees and forests.

Not accounting for circadian rhythms into gas exchange models does not imply that current models will provide a ‘wrong’ answer. The circadian clock has a temporal pattern that will be correlated with the temporal cues of the environmental

drivers. Therefore, any model that considers variation in such environmental drivers is indirectly incorporating circadian regulation. The problem is that the models may be providing a ‘good’ answer for a partly ‘wrong’ reason, as they will be attributing the full response to direct environmental responses, whereas in reality the response is driven by the interaction between such direct responses and the circadian clock (Resco de Dios et al., 2012). A major implication of missing key processes within land-atmosphere models may be limited predictive power, at least on a diurnal scale, under the novel environmental conditions expected under global change.

#### **Effects of circadian regulation on gas exchange in the field**

7. At this point in the discussion it is fair to ask why circadian regulation should be examined in ecosystems and in field settings and what evidence indicates that it actually is an important process. It seems trivial that there will be an overwhelming effect of radiation as the primary driver of the diurnal pattern of  $A$  and  $g_s$  (i.e.: photosynthesis cannot occur in the absence of light), with temperature, vapor pressure deficit and other environmental drivers playing a secondary role. The first, and arguably foremost, reason why circadian regulation is important is that it serves as a “control” for studies on the environmental dependence of gas exchange, which assume that only direct physiological responses are involved. A basic aspect of experimental manipulations is to compare results against a background “null model”. When one is interested in understanding how environmental variation affects gas exchange, responses should be compared against a background where there is no temporal variation in the environment. As previously discussed, experiments in the free-run show how 15-25% of the daytime pattern in  $A$ , and 30-53% of the daytime pattern in  $g_s$ , may be explained without environmental change at a given light level

(Fig. 3). While radiation remains the primary driver of diurnal gas exchange, the magnitude of these circadian effects over  $A$  and  $g_s$  is therefore similar to that of secondary drivers such as temperature and vapor pressure deficit.

A consequence of circadian regulation is that it will affect current studies on the environmental dependence of gas exchange. These studies often rely on either ramped response functions, which study the response of gas exchange to step environmental changes, or on the temporal co-variation between fluxes and drivers during a day, where diurnal changes in flux rates are correlated against environmental variation. The latter approach is often applied to assess ecosystem responses. These two approaches are considered equivalent but, while circadian regulation will not interfere with ramped curves (the environment is rapidly modified), it might affect studies on diurnal co-variation, because the passage of time elicits circadian effects. In fact, a recent study that examined the effects of temperature and vapor pressure deficit on canopy  $A$  in bean and cotton observed how the results of response curves were 14% higher than the results from natural co-variation methods, and the difference was attributed to circadian effects (Resco de Dios et al., 2017). Additionally, significant circadian regulation could also lead to significantly different ramped response curves, depending upon the time of day when the curves were conducted.

There are additional observations in the literature that are difficult to explain unless circadian regulation is invoked. For instance, for a given level of light, net ecosystem exchange of  $\text{CO}_2$  in a tropical forest from Brazil significantly varied in the early morning such that rates could be even two-fold higher from one hour to the next (Doughty et al., 2006). Such variation cannot be explained by other processes such as the kinetics of Rubisco activation, as response times are often smaller than 30

minutes. Similarly, circadian regulation was necessary to explain 24-h gas exchange rhythms in species from the arctic tundra (Patankar et al., 2013), and there are cases where modeling needed to incorporate circadian effects to explain temporal patterns (Mendes and Marenco, 2010; Price and Black, 1989).

Additionally, many species show sharp declines in  $A$  and  $g_s$  in the afternoon (Greaves and Buwalda, 1996; Lüttge and Hertel, 2009; Resco et al., 2008), which could be due to circadian gating. Similarly, diurnal transpiration shows a hysteresis such that, for a given level of vapor pressure deficit, flux rates are higher in the morning than in the afternoon (Matheny et al., 2014; O'Grady et al., 1999). This process is not well understood and it could be partly driven by the asymmetry between radiation and vapor pressure deficit (it is often “darker” in the afternoon for a given level of vapor pressure deficit), and also partly based upon hydraulic signals and depletion of stem capacitors (Zhang et al., 2014). However, circadian gating of stomata, that increases ABA sensitivity in the afternoon remains an alternative yet untested hypothesis.

Circadian effects are particularly important to drive nocturnal transpiration, and they have been estimated to drive 23-56% of the nocturnal variation in *Eucalyptus globulus* (Resco de Dios et al., 2013a). Moreover, circadian controls have been documented to be more important than vapor pressure deficit as a driver of nocturnal  $g_s$  (Resco de Dios et al., 2013b).  $g_s$  often responds negatively to vapor pressure deficit, such that stomata open when vapor pressure deficit decreases. This could partly explain why nocturnal stomatal conductance is higher at predawn, when vapor pressure deficit is minimal. However, in some species including trees, and contrary to the daytime trend,  $g_s$  responds positively to vapor pressure deficit, such that  $g_s$  should be minimal at dawn (Caird et al., 2007; Resco de Dios et al., 2013a). In

these cases it has been demonstrated how  $g_s$  continues to raise over night, despite the negative effect of declining vapor pressure deficit, indicating that circadian regulation may, under some circumstances, be more important than direct physiological responses to vapor pressure deficit (Resco de Dios et al., 2013a).

## **8. Adaptive potential of circadian regulation in gas exchange**

Early studies considered circadian resonance in photosynthesis, whereby photosynthetic rhythms are finely tuned to match environmental cues, as one of the main reasons explaining why circadian regulation is adaptive (Pittendrigh, 1981). This is because circadian resonance allows the plant to anticipate predictable environmental cues, such as dawn or dusk, and to consequently prepare in advance. Indeed, Dodd et al. (2005) found that short and long day *Arabidopsis* mutants showed higher C assimilation rates and growth over short and long days, respectively. This adaptive potential has also been shown for trees: Resco de Dios et al. (2016b) observed how *Eucalypt camaldulensis* genotypes with higher predawn  $g_s$  values responded faster to morning light inputs and showed enhanced C uptake early in the morning and, ultimately, higher biomass accumulation.

However, other studies have documented significant natural variation in circadian period within plant populations. Salmela et al. (2016) observed differences in period of up to 3.5h for different families of *Boechera stricta* that coexist within the same populations growing in Wyoming. Using RILs of *Brassica rapa*, significant genetic correlations between circadian period and  $g_s$  or A have been obtained (Edwards et al., 2011; Yarkhunova et al., 2016). The underlying mechanisms remain unknown, but natural variation in circadian period may an important mechanism for the maintenance of population genetic diversity.

A conservative water use is often considered as advantageous. Indeed, one of the prevailing views underlying stomatal behavior is that they operate to the point where C gain is maximized per unit water lost (Cowan and Farquhar, 1977). This hypothesis has been challenged by more recent propositions that plants seek to maximize C uptake, rather than to optimize water use efficiency (Wolf et al., 2016). There are no tests published in the peer-reviewed literature, to the best of our knowledge, on whether circadian regulation leads to an optimal stomatal behaviour or to C gain maximization. However, our review favors the view that circadian regulation leads towards C maximization. This is, on the one hand, because circadian-enhancement of nocturnal water losses does not represent a conservative water use strategy. On the other hand, circadian-driven changes in chlorophyll composition point towards a strategy that prioritizes C assimilation over photoprotection (García-Plazaola et al., 2017). However, we need stronger quantitative tests on whether circadian regulation is adaptive by either optimizing or maximizing C uptake.

Heterosis, where the performance of a hybrid is superior to that of the parents, has been related to circadian regulation (Ni et al., 2009). In fact, enhanced growth in *Arabidopsis* hybrids has been related to higher photosynthetic activity regulated, amongst other processes, by the effects of CCA1/LHY on chlorophyll content (Ni et al., 2009). The generality of these results for other species including trees remains unknown as, for example, heterosis in *Coffea arabica* has been related to thermotolerance rather than circadian regulation, but this highlights another area where circadian regulation in gas exchange may enhance plant growth and ultimately fitness (Ni et al., 2009).

Most of the studies on circadian gas exchange have been conducted under stress-free conditions and there is an overall lack of data on how circadian regulation

in gas exchange is affected by stress. Hagemeyer and Waisel (1987) observed that circadian periodicity in  $g_s$  was not affected by salinity in *Tamarix aphylla*, but the amplitude of the response was diminished. Habte et al. (2014) argued that, in barley, circadian regulation was a weak driver of photosynthetic responses under osmotic stress and (Greenham et al., 2017) showed that nocturnal  $g_s$  is lower at the early stages of drought in *Brassica rapa*. Others have argued that circadian regulation in gas exchange should be stronger in arid environments (Resco de Dios et al. 2016a). In fact, the window of time for C assimilation in deserts is reduced to the first hours of the morning and, consequently, an endogenous timer that leads to maximal photosynthesis at that time has been proposed to be advantageous. Indirect support from this hypothesis comes from studies on nocturnal water losses (Ogle et al., 2012), which often show relatively high  $g_s$  in these environments overnight which could enhance early morning CO<sub>2</sub> uptake.

## 9. Conclusions

Although the discovery of circadian rhythms predates that of photosynthesis, there is an overall lack of tests on the influence of circadian rhythms in gas exchange in the field. We have summarized a growing body of evidence that points towards circadian regulation as an important driver of diurnal gas exchange in plants. In particular, the observation that circadian regulation could interfere with current approaches to infer the environmental dependence of gas exchange deserves further tests. Furthermore, circadian regulation in  $g_s$  shows a larger amplitude than in  $A$ , indicating a potentially larger role of circadian regulation as a driver of water, than of carbon, cycling. Although lack of data across phylogenies, particularly for gymnosperms, and across different environmental conditions precludes any



generalization on the importance of circadian regulation, current evidence indicates that widespread assessments on the influence of circadian rhythms over diurnal gas exchange in ecosystems should be at the forefront of our research efforts. Overall, we need more studies conducted in species other than *Arabidopsis* and crops because plants that have not been subjected to domestication or that have long woody stems are likely to respond very differently. Since forests are the most important terrestrial carbon sink(Pan et al., 2011), a better knowledge on the mechanisms that influence water loss and carbon gain in these ecosystems that also allows extrapolation to future climatic conditions is indispensable. Progress on the assessment of circadian regulation of photosynthesis and transpiration will depend on the capacity to develop methods that can disentangle direct physiological responses from endogenous circadian regulation, and on the capacity of the scientific community to incorporate and test these novel concepts for the development of a gene-to-ecosystem approach.

## **Acknowledgements**

VRD is funded by the Spanish Government (RYC-2012-10970). We acknowledge useful discussions with many colleagues over the years, including A Hall, J Hartwell, M Goulden, DT Tissue, J Roy and many others.

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1079

1080 **Table 1:** Studies documenting significant (✓) or non-significant (✗) circadian  
1081 regulation in rates of photosynthesis (A), in chlorophyll fluorescence (Fluor) or in  
1082 stomatal conductance ( $g_s$ ). Trees and shrubs are indicated with bold letters.

Source	Species	Functional type and biome	A	Fluor	$g_s$
Mendes and Marengo, (2014)	<b><i>Amphirrhox surinamensis</i></b>	Tropical tree	✓		✓
Dodd et al., (2004); Litthauer, et al., (2015); Gould et al., (2009); Somers et al., (1998); Salomé et al., (2002)	<i>Arabidopsis thaliana</i>	C3 herb	✓	✓	✓
Pallas et al., (1974)	<i>Arachis hypogaea</i>	Legume crop	✓		✓
Patankar et al., (2013)	<b><i>Betula nana</i> L.</b>	Arctic shrub	✓	✓	✓
Edwards et al., (2011); Yarkhunova et al., (2016)	<i>Brassica rapa</i>	Herbaceous crop	✓	✓	✓
Gould et al., (2009)	<i>Capsella bursa-pastoris</i>	Temperate herb		✓	
Patankar et al., (2013)	<b><i>Carex bigelowii</i> Torr.</b>	Arctic shrub	✓	✓	✓
Patankar et al., (2013)	<b><i>Cassiope tetragona</i></b>	Arctic shrub	✓	✓	✓
Chia-Looi and Cumming, (1972)	<i>Chenopodium rubrum</i>	Temperate herb	✓		
Doughty et al., (2006)	<b><i>Chimarris turbinata</i></b>	Tropical shrub	✗		✗
Lüttge and Hertel, (2009)	<b><i>Clusia minor</i></b>	Tropical tree	✓		
Dakhiya et al., (2017)	<i>Coleus blumei</i> ,	Herbaceous crop		✓	
Doughty et al., (2006)	<b><i>Copaifera duckei</i></b>	Tropical tree	✓		
Doughty et al., (2006)	<i>Derris amazonica</i>	Tropical liana	✓		
Doughty et al., (2006)	<b><i>Distachya huber</i></b>	Tropical tree	✗		✓
Doughty et al., (2006)	<b><i>Duguetia flagellaris</i></b>	Tropical shrub	✓		
Patankar et al., (2013)	<i>Eriophorum vaginatum</i> L.	Arctic shrub	✓	✓	✓
Doughty et al., (2006)	<b><i>Eschweilera amazonica</i></b>	Tropical tree	✓		
Resco de Dios et al., (2016b)	<b><i>Eucalyptus camaldulensis</i></b>	Temperate tree			✓
Resco de Dios et al., (2013a)	<b><i>Eucalyptus globulus</i></b>	Temperate tree			✓
Resco de Dios et al., (2013b)	<b><i>Eucalyptus tereticornis</i></b>	Temperate tree			✓
Pavlovic et al., (2009)	<b><i>Ginkgo biloba</i></b>	Temperate gymnosperm		✓	
Sinclair et al., (2008)	<i>Glycine max</i>	Legume crop			✓
García-Plazaola et al., (2017); Marengo et al., (2006); Resco de Dios et al., (2016a)	<b><i>Gossypium hirsutum</i></b>	Shrubby crop	✓	✓	✓
Dakhiya et al., (2017); Deitzer and Frosch, (1990); Gould et al., (2009); Habte et al., (2014)	<i>Hordeum vulgare</i>	Grass crop	✓	✓	✓
Gould et al., (2009); Moriyuki and Fukuda, (2016)	<i>Lactuca sativa</i>	Herbaceous crop		✓	
Doughty et al., (2006)	<b><i>Lecythis lurida</i></b>	Tropical tree	✓		
Doughty et al., (2006)	<b><i>Lecythis</i> sp.</b>	Tropical shrub	✗		✓

Patankar et al., (2013)	<b><i>Ledum palustra L.</i></b>	Arctic shrub	✓	✓	✓
Hillman, (1971)	<i>Lemna perpusilla</i>	Temperate herb	✓		
Corlett et al. (1998)	<b><i>Lycopersicon esculentum</i></b>	Shrubby crop	✓		✓
Allen et al., (2000)	<b><i>Mangifera indica</i></b>	Tree crop	✓	✓	✓
Doughty et al., (2006)	<b><i>Manilkara huber</i></b>	Tropical tree	✓		
Doughty et al., (2006)	<b><i>Micropholis sp.</i></b>	Tropical tree	✓		
Joo et al., (2017)	<i>Nicotiana attenuata</i>	Herbaceous crop	✓		✓
Dakhiya et al., (2017)	<i>Petunia x atkinsiana,</i>	Herbaceous crop		✓	
García-Plazaola et al., (2017); Hennessey et al., (1993); Mencuccini et al., (2000)	<i>Phaseolus vulgaris</i>	Herbaceous crop	✓	✓	✓
Doughty et al., (2006)	<i>Prionostemma aspera</i>	Tropical liana	✗		✓
Doughty et al., (2006)	<b><i>Protium punctulatum Macbr.</i></b>	Tropical tree	✗		✗
Patankar et al., (2013)	<b><i>Salix pulchra Cham.</i></b>	Arctic shrub	✓	✓	✓
Williams and Gorton, (1998)	<b><i>Sarurus cernuus</i></b>	Temperate shrub	✓		✓
Doughty et al., (2006)	<i>Serjania sp.</i>	Tropical liana	✗		✓
Velez-Ramirez et al., (2017)	<i>Solanum lycopersicum L.</i>	Herbaceous crop		✓	
Hagemeyer and Waisel, (1987)	<b><i>Tamarix aphylla</i></b>	Shrub			✓
Patankar et al., (2013)	<b><i>Vaccinium vitis-idaea L</i></b>	Arctic shrub	✓	✓	✓
Gorton et al., (1993)	<i>Vicia faba</i>	Legume crop	✓		✓
Dakhiya et al., (2017); Gould et al., (2009); Ko et al., (2016)	<i>Zea mays</i>	C4 grass crop	✓	✓	✓

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**Figure 1:** Structure of the circadian clock. (a) 24-h oscillations in C assimilation ( $A$ ) or stomatal conductance ( $g_s$ ) are often observed in the free-run (continuous illumination). (b) the classical view of the circadian clock includes inputs, the central oscillator, and outputs. (c) simplified view of the structure of the transcriptional-translational feedbacks that form the central oscillator (modified from (Hernando et al., 2017) and Millar (2016)). Arrows indicate activation, lines with a flat head indicate inhibition.

**Figure 2:** Models of circadian regulation on stomatal aperture. Modified from Hubbard and Webb (2015), Legnaioli et al (2009) and Ando et al. (2013). ELF3: EARLY FLOWERING 3, FT: FLOWERING LOCUS T; TSF: TWIN SISTER OF FT; CO: CONSTANS, GI: GIGANTEA; CCA1: CIRCADIAN CLOCK ASSOCIATED 1; LHY: LATE ELONGATED HYPOCOTYL; TOC1: TIMING OF CAB EXPRESSION 1; SnRK: SNF-1-RELATED KINASE; PP2C Protein phosphatase 2C; ABAR: ABA receptor. Arrows indicate activation, lines with a flat head indicate inhibition.

**Figure 3:** Patterns of carbon assimilation ( $A$ ), stomatal conductance ( $g_s$ ) and intrinsic water use efficiency ( $W_i$ ) under continuous light conditions for the species in Table 1 which reported values starting in early morning. Values include the herbaceous species *Arabidopsis thaliana* (Dodd et al., 2005), *Arachis hypogaea* (Pallas et al. 1974), *Hordeum vulgare* (Deitzer and Frosch, 1990), *Lycopersicon esculentum* (Corlett et al., 1998), *Phaseolus vulgaris* (García-Plazaola et al., 2017), *Saururus cernuus* (Williams and Gorton 1998), the perennial shrub *Gossypium hirsutum* (García-Plazaola et al., 2017) and, and the tree species *Mangifera indica* (Allen et al.,

2000). Plotted values represent normalized  $A$ ,  $g_s$  and  $W_i$  (by dividing each value by the maximum value for that species) measured in the free-run after an entrainment regime of 12 hours of day and of night (except Allen et al. 2000, where daylength was 11 hours). Modified from Resco de Dios (2017).

**Figure 4:** Experimental facilities for studying circadian assimilation in ecosystems. The CNRS Ecotron in Montpellier, FR, (a, b) and the whole tree chambers from Western Sydney University, AU, (c) allow for precise environmental control and on-line gas exchange measurements. Eddy covariance towers, such as the one from University of Castilla-la Mancha, ES (d), allow for continuous estimates of net ecosystem  $\text{CO}_2$  and  $\text{H}_2\text{O}$  exchange that may be used to infer circadian regulation.

**Figure 5.** Conceptual example of a simplified empirical scheme to incorporate circadian regulation within ecosystem flux studies. In the traditional approach, examining the response of  $\text{CO}_2$  exchange ( $F_c$ ) to variations in air temperature ( $T_{\text{air}}$ ), is done after examining the temporal co-variation between  $F_c$  and different  $T_{\text{air}}$  classes (B, or also with raw values), under different Photosynthetically Active Radiation (PAR) ranges (A, here we chose  $\text{PAR} > 1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$  it had a negligible effect over  $F_c$ ;  $R^2 = 0.009$ ,  $P = 0.005$ ) and over a few weeks to avoid seasonal effects. Under this approach, we would observe optimum  $T_{\text{air}}$  between 22.5 and 27.5 C, and a large decrease occurring thereafter (B). This approach is problematic as it assumes that temporal co-variation between fluxes and environmental drivers may replace response curves. We suggest the additional inclusion of solar time within this framework, which then leads to the observation of non-significant differences to  $T_{\text{air}}$  under  $<37.5\text{C}$  for some parts of the day (simplifying, this is indicated by the overlap in the error

bars, which indicate the 95% CI, in C). This approach can be then expanded to test whether this is due to changing air water vapour pressure deficit (VPD) through time within a  $T_{\text{air}}$  bin (D). In this simplified example, which does not take into account changes in solar angles, we observed a significant time x VPD x  $T_{\text{air}}$  interaction, indicating  $F_c$  dependencies over environmental drivers changed through time. While this analyses of the drivers of  $F_c$  is overly simplistic, we include this example simply for illustrative purposes. More accurate and realistic analyses can be performed after inclusion of endogenous oscillators in process-based or statistical models, as presented in the text. It is noteworthy that, beyond circadian regulation, other processes such as carbohydrate accumulation and Rubisco inhibition (Azcón-Bieto, 1983), reductions in hydraulic capacity (Jones, 1998) or afternoon increases in photorespiration, also create differential temporal responses in  $A_c$  or  $E_c$ . This time-binning approach would not partition between circadian regulation and other endogenous processes, but it would additionally consider them. Eddy covariance data was collected at the Cumberland Plains Woodland Ozflux tower in SE Australia (data available through Fluxnet). The canopy of the forest is dominated by *Eucalyptus moluccana* and *Eucalyptus fibrosa* with a maximum height of 23 m.  $F_c$  axis has different scales in different panels.

Figure1

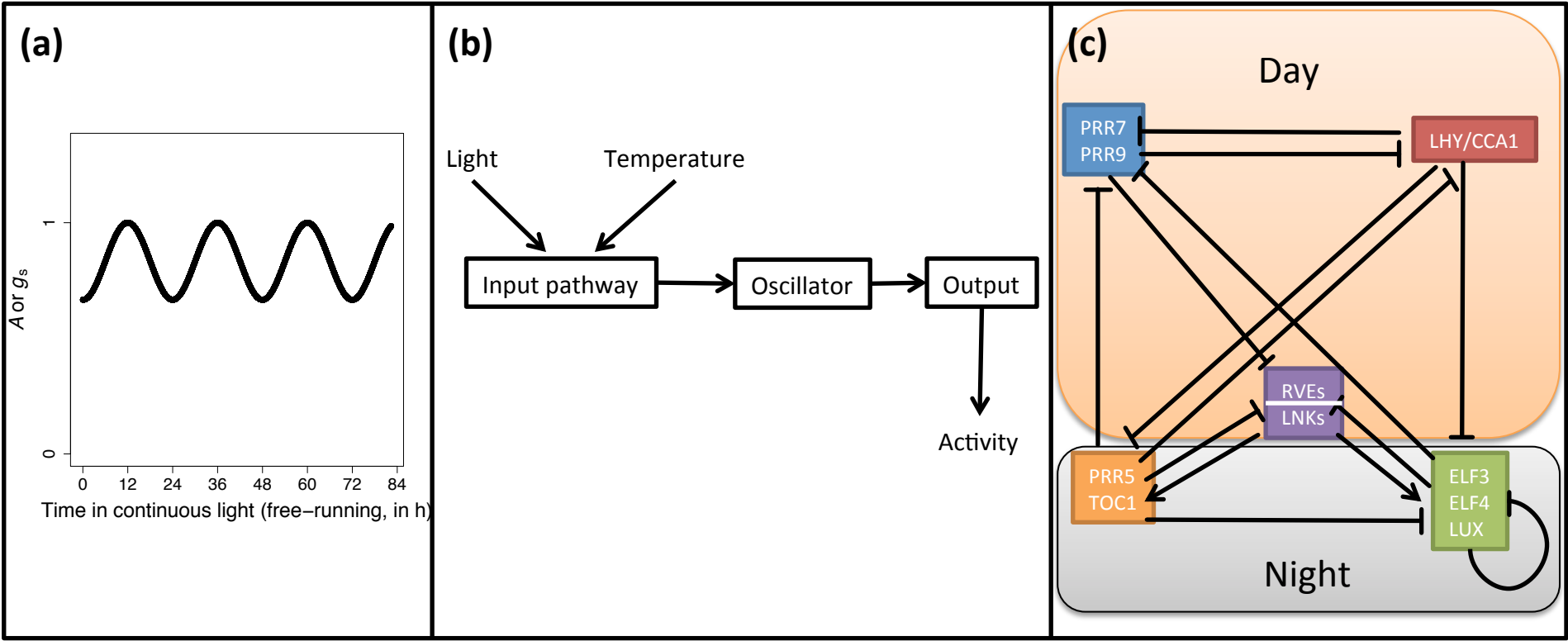


Figure2

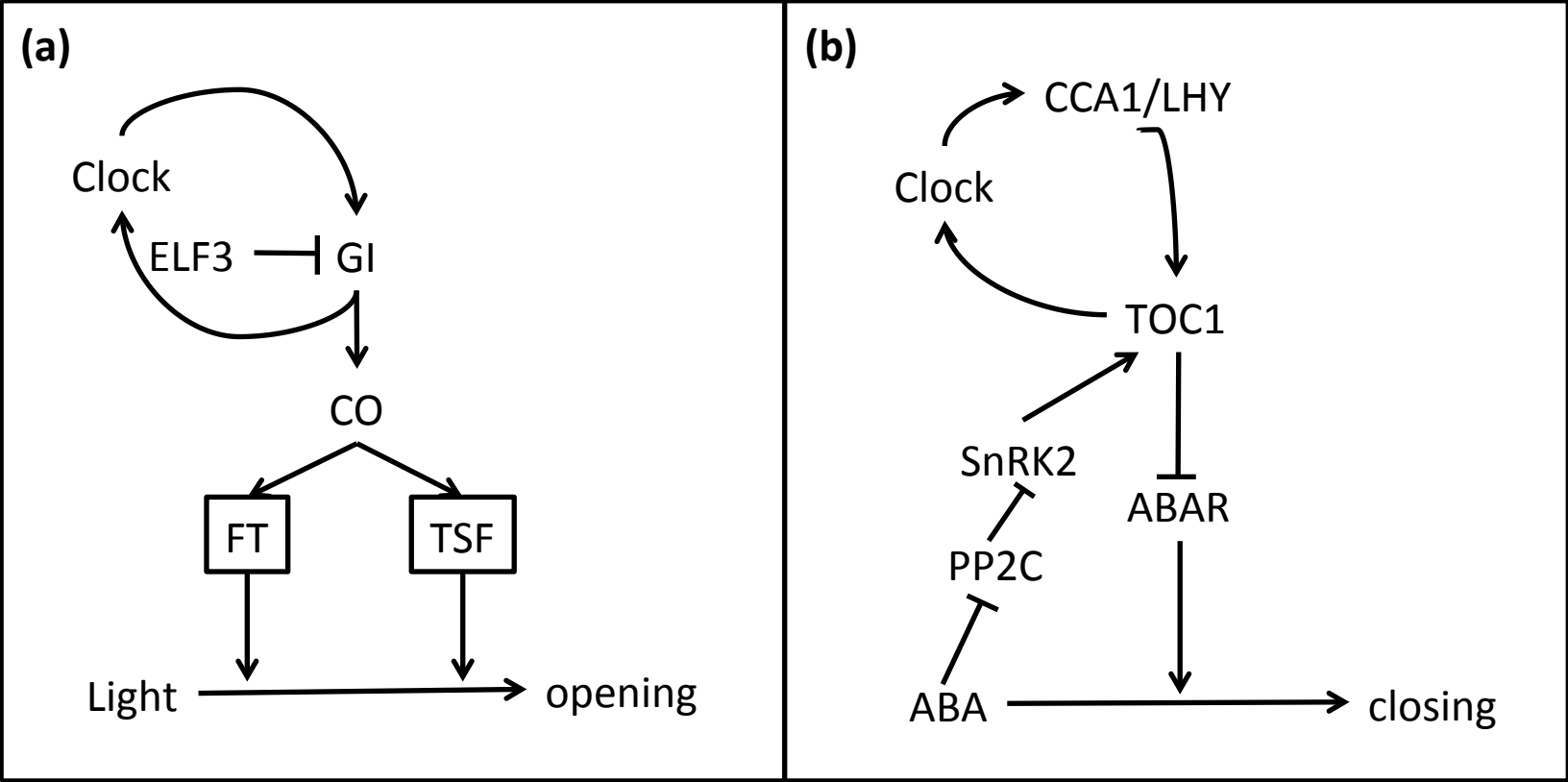




Figure3

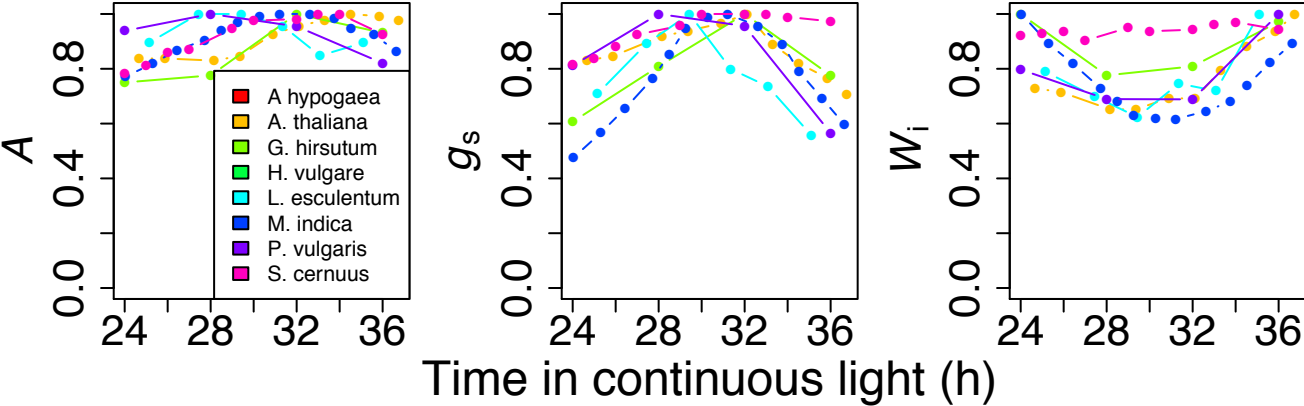


Figure4

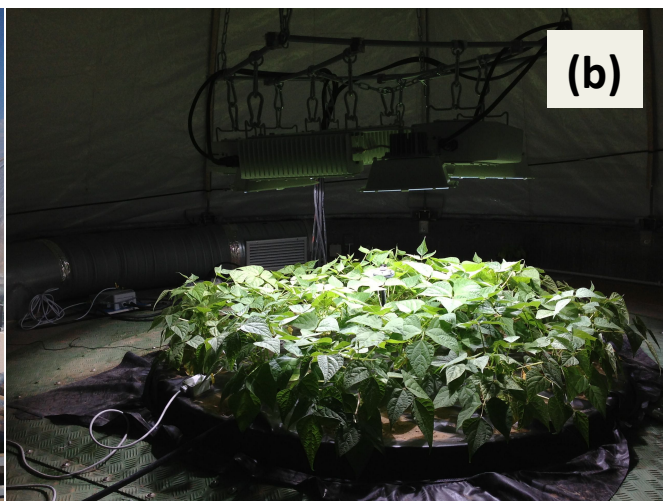


Figure5

